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1st Cancer Update

In vivo anticancer and histopathology studies of Schiff bases on Ehrlich ascitic carcinoma cells

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Abstract Three Schiff bases in two different concentrations were evaluated for their anti-tumor activity against Ehrlich ascites carcinoma (EAC) bearing Swiss albino mice. The *in vivo* anti-tumor potency of Schiff bases was assessed by measuring the increase in mean survival time of the drug treated over untreated control mice and treated standard (cisplatin) mice. Their toxicity was assessed *in vivo* in normal, standard, and EAC-bearing mice by measuring the drug-induced changes in biochemical as well as hematological parameters. The histopathology studies to assess the toxicity of these compounds on vital organs also have been studied. Among the three Schiff bases studied, 4-([3-(4-fluorophenyl)-1H-pyrazol-4-yl]methylene)amino-5-[(2-methylphenoxy)methyl]-1,2,4-triazole-3-thiol (SB-3) at an optimal dose of 100 mg/kg body weight was found to enhance the mean survival time of infected mice. Deviated hematological parameters and mean survival time in tumor

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bearing mice were found to be significantly restored towards normal after treatment with SB-3 100 mg/kg body weight of mice. The ALP and SGOT values were found to approach the normal range. A:G ratios also did not deviate from normal on treatment with SB-3. The histopathology studies revealed only mild hepatotoxicity and nephrotoxicity when compared to the normal and standard. The splenic cellularity also did not show much variation from normal. SB-3 at a prime dose of 100 mg has shown promising anticancer activity *in vivo* against EAC when compared to standard drug with minimum toxic effects.

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1. Introduction

Cancer or malignant neoplasm is a class of diseases in which a group of cells display uncontrolled growth, invasion and even sometimes metastasis (De Vita et al., 2005; Thomas and Vinay, 2007). It continues as a serious public health problem throughout the world as the most feared diagnosis. It is the second leading cause of human death after cardiovascular diseases in developing as well as in developed countries (Babasaheb et al., 2010). Currently, the treatment for cancer primarily includes surgery and chemotherapy, but the curative effects of the existing chemotherapeutic drugs are not good enough and they have plentiful side effects. The development of more effective drugs for treating patients with cancer has been a main attempt over the past 50 years. In recent years, various 1,2,4-triazole derivatives have been found to be associated with anticancer (Al-Soud et al., 2006; Holla et al., 2002; Ikizler et al., 2003) properties. AK-2123 (Sanazol), a nitrotriazole hypoxic cell sensitizer has supposedly improved results in head and neck cancers, uterine cervical cancers and other solid tumors when added to radical radiotherapy (Dobrowsky et al., 2005). Non-steroidal aromatase inhibitors obtained from triazole derivatives are used in the treatment of breast cancer (Robert et al., 2004). Current literature shows 1,2-pyrazole derivatives to possess various biological activities (Mathew et al., 2007; Konagai et al., 2002). It is also observed that incorporation of aryl substituents and halogen atoms into the heterocyclic ring systems enhances the biological activities considerably (Padmavathi et al., 2009). Several Schiff bases were reported to possess potential anticancer properties (Holla et al., 2003). In view of the wide spectrum of medicinal applications, especially anticancer properties of various triazole derivatives and in continuation of our research on biologically active new molecules, we hereby report the anticancer activity of three Schiff bases *in vivo*.

In vitro screening in hepatic carcinoma cell lines (Hep G2) using MTT assay, revealed the cytotoxicity of the Schiff bases (Dhanya et al., in press). Their *in vivo* anti-tumor potency was assessed in Ehrlich ascites carcinoma (EAC) cells by assessing the increase in median survival times and body weights of drug treated (T) over untreated control (C) and standard (S) mice. Results showed that the survival time of treated mice was markedly increased by SB-3 at an optimal dose of 100 mg/kg body weight. Its toxic effects was studied *in vivo* in normal and EAC-bearing mice by measuring drug-induced changes in hematological, biochemical parameters as well as organ toxicities on day 15 following drug treatment. The hematological and biochemical parameters were mostly within normal limit. Histology of liver and kidney revealed mild abnormality upon treatment with SB-3 (100 mg/kg body weight). Minor splenic toxicity in treated groups SB-3 (100 mg/kg body weight) similar to standard group was observed.

2. Materials and methods

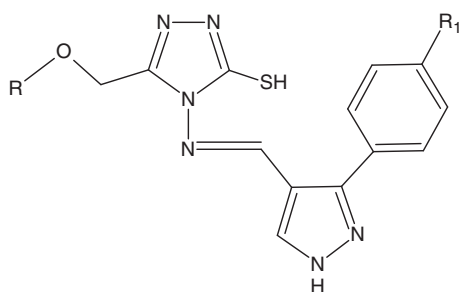
All the chemicals used in the present study were from Sigma-Aldrich, USA. Adult female swiss albino mice of 6–8 weeks old weighing 25–30 g, inbred at Central Animal Research Facility, Manipal University, Karnataka, India were used throughout the study. Animals were housed in polypropylene cages containing sterile paddy husk as bedding material under hygienic conditions with a maximum of four animals in a cage. They were maintained under controlled conditions (10:14 h light:dark), temperature ($23 \pm 3^\circ\text{C}$). Animals were fed on auto-claved standard mice food pellets (Hindustan Lever) and water *ad libitum*. The animal experiments were performed according to the rules and regulations of the Institutional Animal Ethics Committee (IAEC).

Three Schiff bases, 4-([3-(4-chlorophenyl)-1H-pyrazol-4-yl]methylene)amino-5-[(4-methylphenoxy)methyl]-1,2,4-triazole-3-thiol (SB-1), 4-([3-(4-fluorophenyl)-1H-pyrazol-4-yl]methylene)amino-5-[(4-methylphenoxy)methyl]-1,2,4-triazole-3-thiol (SB-2) and 4-([3-(4-fluorophenyl)-1H-pyrazol-4-yl]methylene)amino-5-[(2-methylphenoxy)methyl]-1,2,4-triazole-3-thiol (SB-3) were prepared by the method as described in the literature (Kalluraya et al., 2009; Dhanya et al., in press). Their structures were confirmed by IR, NMR and mass spectral studies. IR spectra of Schiff bases showed an absorption band at $1600\text{--}1610\text{ cm}^{-1}$ characteristic of $\text{C}=\text{N}$ group in the molecule. Also the absence of carbonyl stretching band at around 1700 cm^{-1} clearly indicated amino condensation and hence the formation of Schiff bases. The ^1H NMR spectrum of Schiff base showed a singlet at δ 9.95 due to the presence of proton in $\text{N}=\text{CH}$ group in the molecule, confirming the formation of Schiff bases. Mass spectrum of the three Schiff bases showed molecular ion peaks which were in agreement with their molecular formula. The elemental analysis was also carried out. The purity of the compounds was checked using LC-MS technique. The structure of the Schiff bases are given in Fig. 1.

2.1. *In vivo* anticancer studies

The drug solutions were prepared daily just prior to the injection by suspending them in 0.25% CMC (carboxy methyl cellulose) and was administered intraperitoneally. The dose of the standard drug, cisplatin was selected as 3.5 mg/kg mice body weight. This was calculated from the human dose using an appropriate conversion factor (Sathisha et al., 2008).

Ehrlich ascites carcinoma cells to induce cancer in animal model (mice) were obtained from Amala Cancer Research Center, Amala Nagar, Kerala, India. The cells were maintained as ascites tumor in swiss albino mice by intraperitoneal inoculation of 1×10^6 viable cells (Sathisha et al., 2008; Devi



SB-1: R = 4-methyl benzene, R₁ = Cl, SB-2: R = 4-methyl benzene, R₁ = F
SB-3: R = 2-methyl benzene, R₁ = F

Figure 1 Structure of Schiff bases.

et al., 1998). Acute toxicity studies were carried out as per the OECD guidelines, 2001. For determining the maximum tolerated dose of the Schiff bases, the standard protocol was followed by administering the Schiff bases to normal swiss albino mice at the dose range up to 2000 mg/kg body weight after depriving them of food for 18 h. Animals were observed for any symptoms of toxicity continuously for 4 h, then after 24 h and finally the number of survivors were noted after a period of 72 h. Depending on the results obtained, the therapeutic doses for further studies were selected (1/10th to 1/20th of the maximum tolerated dose).

For the *in vivo* assay, nine groups were formed. Each group comprised of 12 female swiss albino mice. Formed groups are as follows:

- Group 1: The normal mice.
- Group 2: The EAC-bearing mice (Control).
- Group 3: The EAC-bearing mice on a single dose of cisplatin (Standard).
- Groups 4–9 represented the study group, treated with Schiff bases as depicted below:
 - Group 4: SB-1 (50 mg/kg).
 - Group 5: SB-1 (100 mg/kg).
 - Group 6: SB-2 (50 mg/kg).
 - Group 7: SB-2 (150 mg/kg).
 - Group 8: SB-3 (50 mg/kg).
 - Group 9: SB-3 (100 mg/kg).

The ascitic carcinoma bearing donor mice was taken 15 days after tumor transplantation. The ascitic fluid was drawn using an 18-gauge needle into a sterile syringe. A small amount was tested for microbial contamination. Tumor viability was determined by Trypan blue exclusion test and cells were counted using haemocytometer. The ascitic fluid was suitably diluted in normal saline to get a concentration of 10×10^6 cells/mL of tumor cell suspension. From this stock suspension, 0.25 mL (2.5 million cells/mice) was injected intraperitoneally to normal mice in order to obtain ascitic tumor (day 0). Cisplatin (single dose) was given intraperitoneally to the standard group on day 1. The three Schiff bases in two different concentrations were administered on days 3, 5, 7, 10, 12 and 14 (6 doses) intraperitoneally. The control group was treated with the vehicle (0.25% CMC). On day 15, from each group six mice were sacrificed for hematological, biochemical and histopathological studies. The remain-

ing six animals from each group were monitored daily for 30 days and mortality was recorded to calculate the mean survival time.

2.1.1. Mean survival time and increase in lifespan (% ILS) (Sathisha et al., 2008)

Total number of days an animal survived from the day of tumor inoculation was counted. Subsequently mean survival time (MST) for each group was calculated. Survival time for treated group was compared with that of control group using the following calculation:

Increase of life span (ILS)

$$= \left[\frac{\text{MST of treated group} - \text{MST of control group}}{\text{MST of control group}} \right] \times 100$$

where $\text{MST} = \frac{\sum \text{survival time (days) of each mice in a group}}{\text{Total number of mice}}$.

2.1.2. Body weight (Eckhardt et al., 1982)

Body weights of all animals were measured on days 0, 3, 5, 7, 10, 12 and 14. The percentage increase in weight was calculated as per the following equation:

% increase in weight

$$= \left(\frac{\text{Animal weight on respective day} - \text{Animal weight on day 0}}{\text{Animal weight on day 0}} \right) \times 100$$

2.2. In vivo toxicological studies

On day 15, blood samples from six animals in each group were taken in microfuge tubes containing EDTA for hematological studies and the serum was collected for biochemical studies. The animals were then sacrificed by cervical dislocation, dissection was done and the liver, kidney and spleen were removed for histopathology studies.

2.2.1. Hematological parameters (Mukherjee et al., 2007)

To study the hematological changes associated with Schiff base administration, four parameters – (a) hemoglobin count (b) erythrocyte count, (c) leukocyte count and (d) differential count were determined in peripheral blood of mice. The blood was withdrawn from retro-orbital plexus on day 15 for the study. The values were then compared with the normal and standard values.

2.2.2. Biochemical parameters (Pain et al., 2003)

Numerous laboratory investigations have been proposed in the assessment of liver dysfunction. For evaluating drug induced hepatotoxicity, liver function tests were done and values obtained for treated groups were compared with the normal value. From among the host of liver function tests, the following battery of blood tests namely bilirubin, SGOT, SGPT, ALP and protein levels were carried out.

2.3. Histopathology studies (Roy et al., 1981; Hassan et al., 2009)

The animals were sacrificed by cervical dislocation. The liver, kidneys and spleen were removed and stored in neutral forma-

lin and histopathology studies were conducted. These were processed by standard methods to prepare slides of tissues by hematoxylin and eosin staining. The slides were viewed under light microscope. Drug induced hepatotoxicity, nephrotoxicity and spleen toxicity studies were performed sequentially.

2.4. Statistical analysis

The statistical analyses were performed by one-way ANOVA, followed by Tukey's post hoc test using GraphPad Prism 5.02. The results were expressed as the mean \pm SEM. Differences were considered significant $p < 0.05$.

3. Results and discussion

Cisplatin (3.5 mg/kg, *i.p.* single dose), significantly enhanced MST of EAC infected mice. Both SB-1 and SB-3 in both con-

centrations were found to increase the survival time of animals. However, SB-3 at an optimal dose of 100 mg/kg showed remarkable enhancement in the survival time of mice. It increased the mean survival time by 24%. SB-2 in both concentrations was found to be inactive. The detailed data showing the mean survival time is shown in Fig. 2.

Substantial gain in body weight was observed in control mice with a maximum gain of 36.12%. In the case of SB-3 (100 mg/kg) treated mice, gain in body weight was only 11.11% indicative of its efficiency in bringing down the progression of cancer. The graph depicting the body weight changes of animals is shown in Fig. 3.

Literature survey reveals that progression of tumor was accompanied by the following hematological changes compared to normal gradual decrease in hemoglobin content, erythrocyte count and gradual increase in leukocytes which was observed in control mice. The RBC count was almost restored back to normal range on treatment with SB-3 (100 mg/kg). It could also improve the RBC count, but not as efficiently

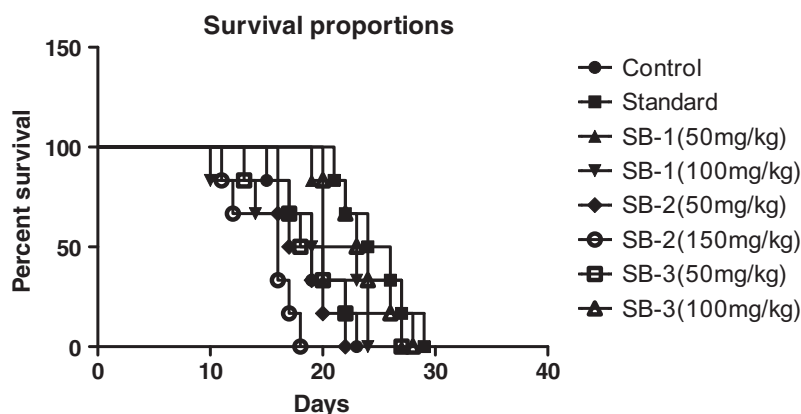


Figure 2 Effect of Schiff bases on the Kaplan Meier's estimate of survival of EAC-bearing mice. After EAC inoculation (2.5×10^6 cells/mouse, *i.p.*), mice were treated with six doses of Schiff bases in two different concentrations and single dose of cisplatin (3.5 mg/kg). Animals were monitored daily for 30 days.

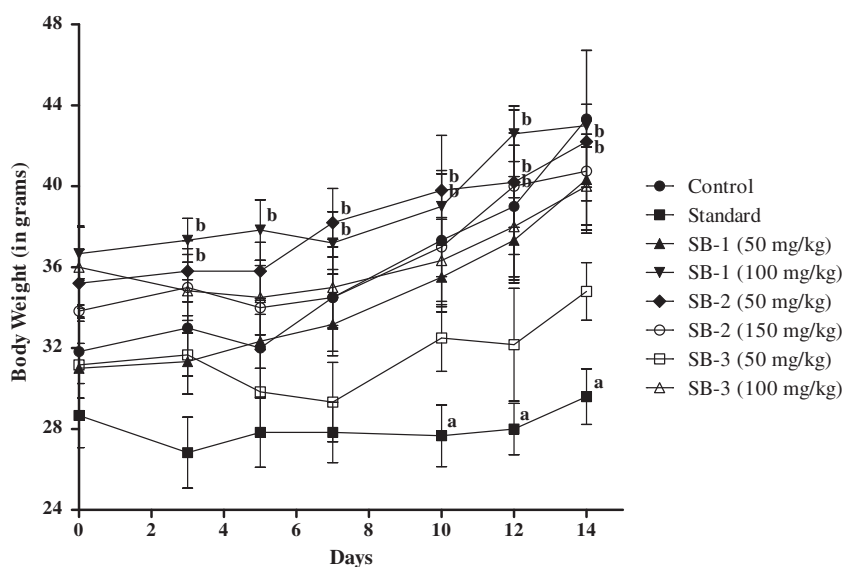


Figure 3 Effect of Schiff bases on body weight changes in EAC inoculated mice. All values are the mean \pm SEM of six mice. ^a $P < 0.05$ compared to control and ^b $P < 0.05$ compared to standard.

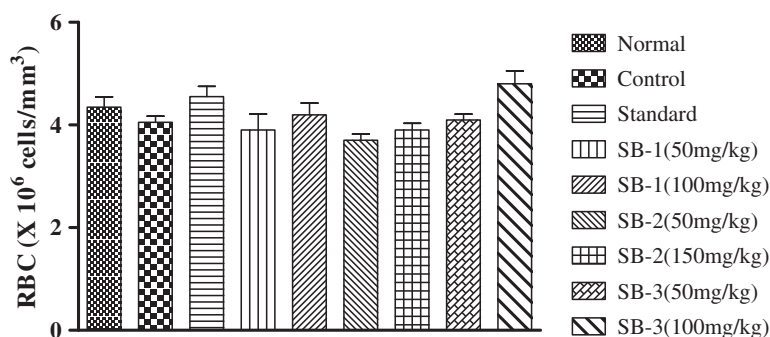


Figure 4 Effect of Schiff bases on RBC count in EAC challenged mice.

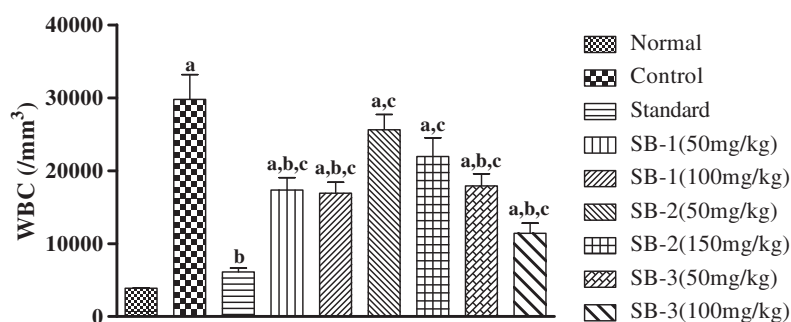


Figure 5 Effect of Schiff bases on WBC count in EAC induced mice. ^a*P* < 0.05 compared to normal, ^b*P* < 0.05 compared to control and ^c*P* < 0.05 compared to standard.

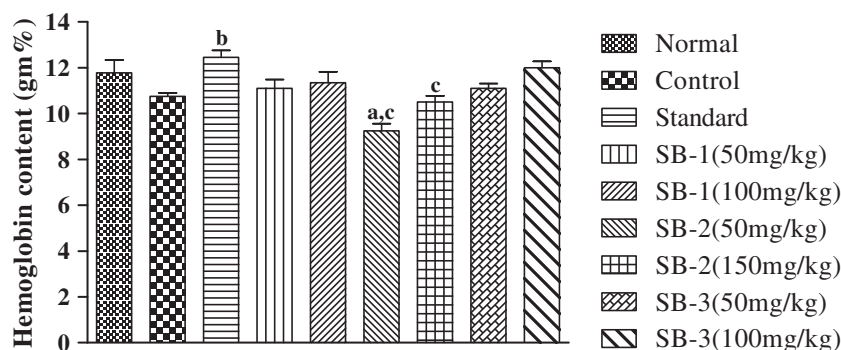


Figure 6 Effect of Schiff bases on hemoglobin count in EAC inoculated mice. ^a*P* < 0.05 compared to normal, ^b*P* < 0.05 compared to control and ^c*P* < 0.05 compared to standard.

as SB-3 (100 mg/kg). The Hemoglobin levels were in the normal range in the SB-3 (100 mg/kg) treated groups. SB-3 at an optimal dose of 100 mg/kg could bring down the WBC level, but not as efficiently as cisplatin. The detailed data obtained for each hematological parameter is given in Figs. 4–6. SB-3 (100 mg/kg) could bring down the count by 22% compared to control.

It is well known that there are significant elevations in the levels of ALP (alkaline phosphatases) in case of chronic liver problems. The ALP values were in normal range in SB-3 (100 mg/kg) treated group which is depicted in Fig. 7. Increases in both transaminases are found in liver diseases, with SGPT (Serum glutamic pyruvic transaminase) much higher than SGOT (Serum glutamic oxaloacetic transaminase). Very

high values are usually obtained in toxic hepatitis. The SGPT and SGOT values remained in the normal range in SB-3 (100 mg/kg) treated group, suggestive of its efficiency with less toxic effects when compared with the standard as seen in Fig. 8. Plasma proteins yield most useful information in chronic liver diseases. Liver is the site of albumin synthesis and also possibly of some α and β globulins. Albumin (A) is grossly decreased and the globulins (G) often increased, so that A:G is reversed, which are characteristically seen in advanced liver diseases. In the case of SB-3 (100 mg/kg) treated mice, the protein levels did not vary significantly from normal. Serum bilirubin is also indicative of hepatotoxicity. The total bilirubin levels were also in the normal range in SB-3 (100 mg/kg) treated mice as shown in Fig. 9.

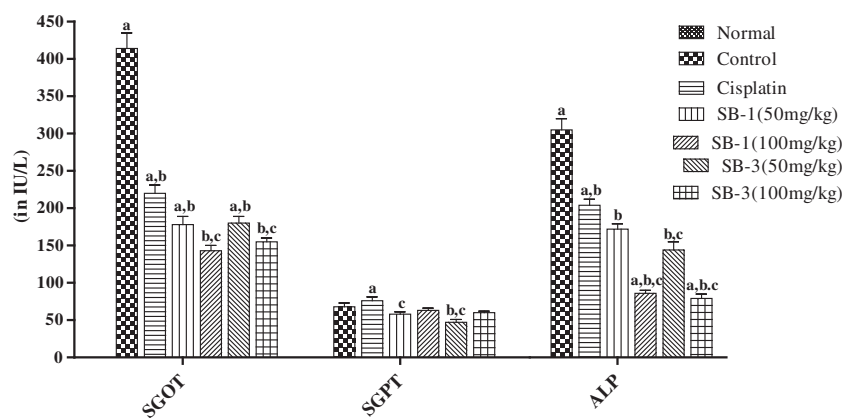


Figure 7 Effect of Schiff bases on transaminases and ALP in EAC challenged mice. ^a $P < 0.05$ compared to normal and ^b $P < 0.05$ compared to control.

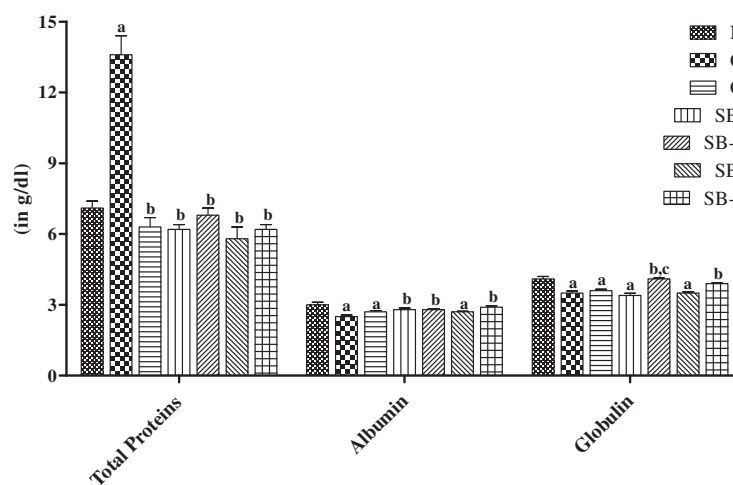


Figure 8 Effect of Schiff bases on protein content in EAC induced mice. ^a $P < 0.05$ compared to normal and ^b $P < 0.05$ compared to control.

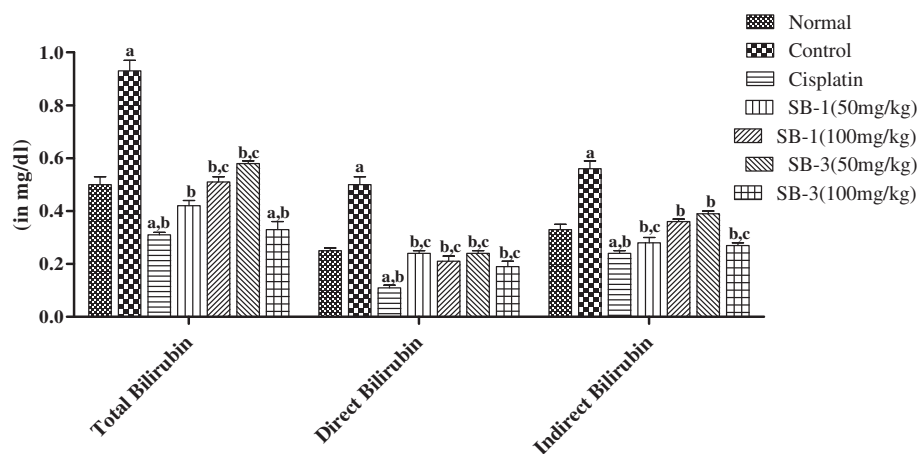


Figure 9 Effect of Schiff bases on Bilirubin count in EAC induced mice. ^a $P < 0.05$ compared to normal and ^b $P < 0.05$ compared to control.

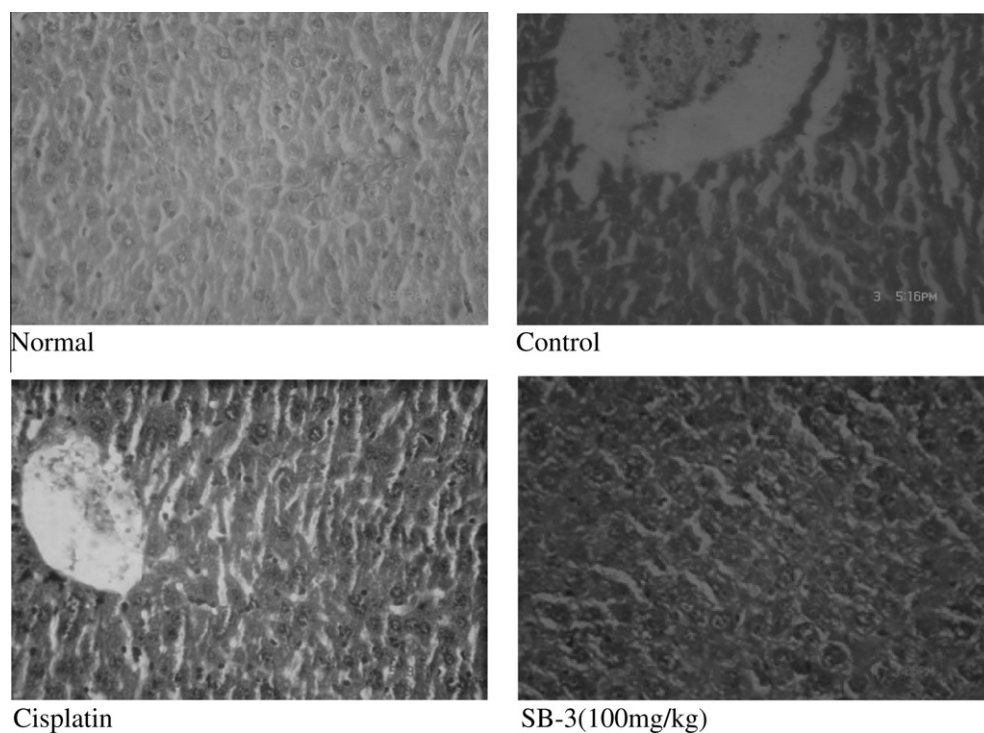


Figure 10 Histopathology of liver (40 \times). Animals were sacrificed on day 15 and histopathology of liver was studied for signs of hepatotoxicity. The histology of liver of standard group showed cellular infiltration, congestion and mild central vein dilatation. SB-3 (50 mg/kg) treated group showed only mild central vein dilation suggesting less hepatotoxicity compared to control and standard.

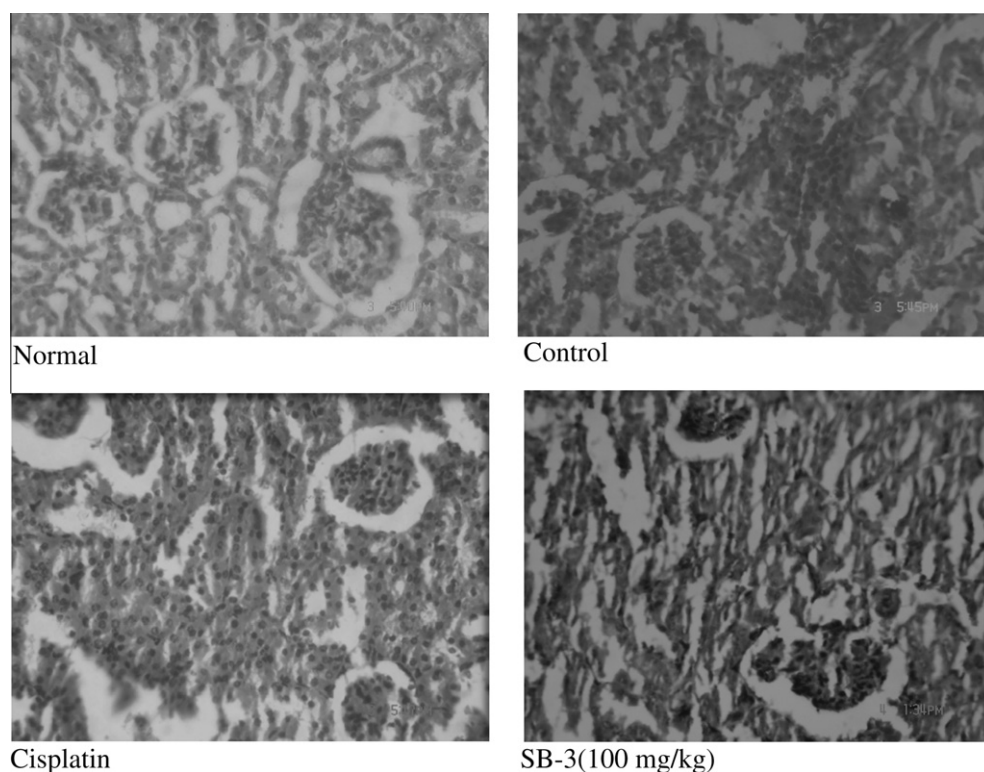


Figure 11 Histopathology of kidney (40 \times). Animals were sacrificed on day 15 and histopathology of kidney was studied for signs of nephrotoxicity. The standard showed signs of nephrotoxicity due to glomerular infiltration. The SB-3 (100 mg/kg) treated group also showed mild glomerular infiltration. There were no signs of tubular necrosis, casts and glomerular congestion, which was indicative of only mild nephrotoxicity with SB-3 (100 mg/kg).

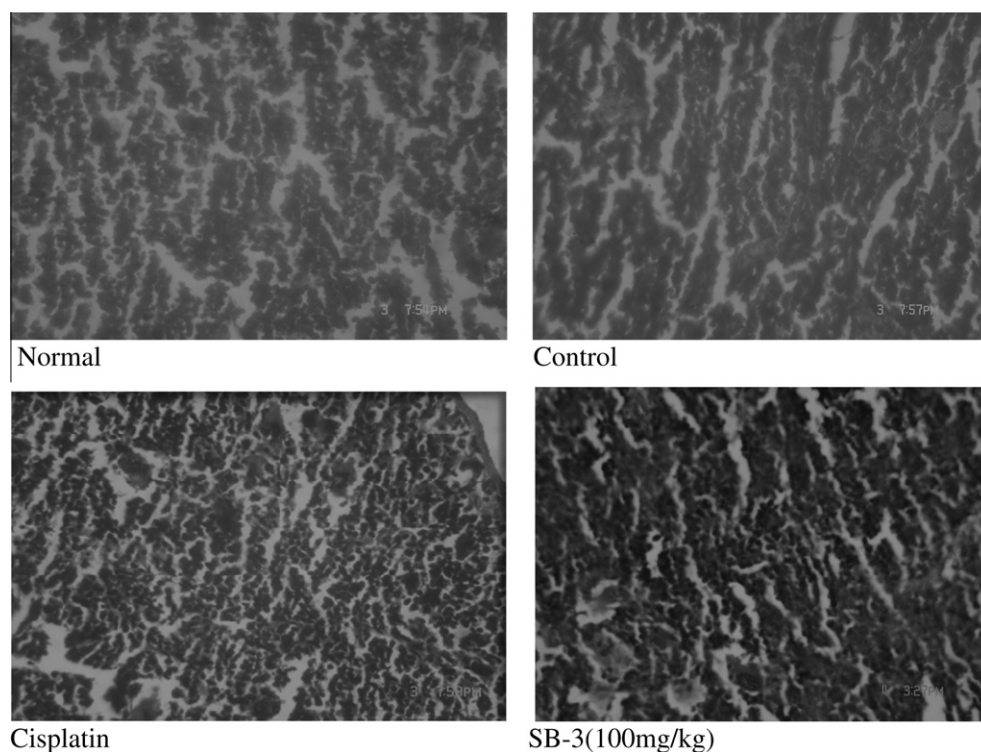


Figure 12 Histopathology of spleen (40 \times). Animals were sacrificed on day 15 and tissue sections of spleen were studied for signs of spleen toxicity. Mild loss in spleen architecture is observed in SB-3 (100 mg/kg) and cisplatin treated mice spleen.

The histopathology studies also revealed the relatively less toxic nature of SB-3 (100 mg/kg) as compared to control and standard group when viewed under light microscope of magnification 40 \times . The liver is known to accumulate significant amounts of cisplatin, second to kidney. The histology studies of liver of standard group showed cellular infiltration (inflammation), congestion and mild central vein dilatation as seen in Fig. 10. Whereas the SB-3 (100 mg/kg) treated group showed only mild central vein dilation suggesting less hepatotoxicity compared to standard group. The histopathology of the kidney tissues of normal, control, standard and SB-3 (100 mg/kg) treated mice are shown in Fig. 11. The control group showed some cellular infiltration, while the standard group showed signs of nephrotoxicity due to cellular and glomerular infiltration. The SB-3 (100 mg/kg) treated group also showed mild glomerular infiltration. There were no signs of tubular necrosis, casts and glomerular congestion, which were indicative of only mild nephrotoxicity, with SB-3 (100 mg/kg). The standard group showed loss of splenic architecture and congestion as depicted in Fig. 12. The treated group also showed mild loss of splenic architecture but congestion was not observed indicating lower splenic toxicity due to treatment with SB-3 (100 mg/kg).

4. Conclusions

In the light of the above observations, it can be concluded that SB-3, at a dose of 100 mg/kg, optimally inhibits the growth of EAC cells *in vivo*. This is evident from the reduced tumor weight and enhanced life span of that study group. The treatment with SB-3 (100 mg/kg) restored the deviated hematological and biochemical parameters to the normal range. It is an

effective antineoplastic agent with comparatively less toxic effects. It is necessary that the anti-tumor activity of this Schiff base should be carried out against other tumor cell lines which may bring promising results in cancer chemotherapy.

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